

A Drug Resistance Mutation in the Inhibitor Binding Pocket of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Impairs DNA Synthesis and RNA Degradation[†]

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ABSTRACT: Selection of the IIIB strain of human immunodeficiency virus type (HIV-1) resistant to the (alkylamino)piperidine-bis(heteroaryl)piperazine (AAP-BHAP) U-104489 results in substitution of a glycine to glutamate at residue 190 (G190E) of reverse transcriptase (RT). The AAP-BHAP resistant HIV-1 displays reduced *in vitro* replication capacity [Olmsted, R. A., et al. (1966) *J. Virol.* 70, 3698-3705]. We report here that the G190E mutation in recombinant heterodimeric HIV-1 RT, compared to the wild-type RT (G190) or a G190A control mutant, results in a 40% and 80% reduction in the polymerase and RNase H specific enzymatic activities, respectively. A primer-extension assay that allowed determination of DNA elongation by the G190E mutant RT on a heteropolymeric HIV-1 gag-based RNA template showed an overall decrease in DNA polymerization. The size distribution of products generated by G190E RT-associated RNase H digestion of RNA from [³⁵S]poly(rA)·poly(dT) was markedly distinct from that of the G190A RT and was consistent with the observed reduction in RT-associated RNase H activity of the G190E RT. When challenged with unlabeled substrates, the G190E RT was relatively nonprocessive with respect to DNA synthesis and RNA degradation. It is concluded that the deleterious effect of the G190E resistance mutation on both of these RT functions is most likely involved in the observed retarded replication capacity of the AAP-BHAP- (U-104489-) resistant HIV-1.

Recent studies on patients infected with human immunodeficiency virus type 1 (HIV-1)¹ have shown an almost complete change from wild-type HIV-1 in plasma to drug-resistant variants after 14 days of drug treatment (Wei *et al.*, 1995; Ho *et al.*, 1995). These results are consistent with the proposed model for the infection process which implies that the wild-type virus remains only for a short period following drug therapy (Coffin, 1995). This relatively new picture of viral dynamics in HIV-1 infected patients suggests new strategies for combating the virus (Wei *et al.*, 1995; Ho *et al.*, 1995; Coffin, 1995). Accordingly, it may be optimal to initiate treatment as early as possible and drugs chosen should be those for which the resistant virus displays retarded growth kinetics.

It is well-known that the first generation of nonnucleoside reverse transcriptase inhibitors (NNRTIs) of HIV-1 share the

same inhibitor-binding pocket (Wu *et al.*, 1991; Dueweke *et al.*, 1992; Sardana *et al.*, 1992; Boyer *et al.*, 1993; Taylor *et al.*, 1994) which is in close proximity to the active site of the p66 subunit of the p66/p51 RT heterodimer (Kohlstadet *et al.*, 1992; Nani *et al.*, 1993; Smerdon *et al.*, 1994). However, a major problem for most of these NNRTIs is the rapid selection of drug-resistant HIV-1 strains in cell culture as well as in HIV-1 infected patients (Nunberg *et al.*, 1991; Richman *et al.*, 1991; Mellors *et al.*, 1992; Balzarini *et al.*, 1992, 1993; Saag *et al.*, 1993; Richman *et al.*, 1994). The resistance mutations stem from codon changes in the RT regions that correspond to amino acids 98-110 and 179-190 of the heterodimeric HIV-1 RT. These drug resistance mutations are well characterized and documented (De Vreese *et al.*, 1992; De Clercq, 1994; Schinazi *et al.*, 1994). The catalytic functions of most mutant RT enzymes closely resemble the wild-type HIV-1 RT (Boyer *et al.*, 1993; Fan *et al.*, 1995; Loya *et al.*, 1994; Bacolla *et al.*, 1993). This includes the substitution of amino acid glycine to alanine at 190 (G190A), which can confer resistance to nevirapine but not to other classes of NNRTIs (Bacolla *et al.*, 1993). However, a unique glycine to glutamate mutation at residue 190 of HIV-1 RT (G190E) was observed in response to treatment of HIV-1-infected cells with the quinoxaline derivative S-2720 (Kleim *et al.*, 1993). Subsequent *in vitro* studies (Kleim *et al.*, 1994) showed that glutamate substitution at G190 in HIV-1 RT is detrimental to its RT activity and sensitivity to NNRTIs.

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¹ Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H, ribonuclease H; IMAC, immobilized metal affinity chromatography; AAP-BHAP, (alkylamino)piperidine bis(heteroaryl)piperazine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TCA, trichloroacetic acid; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PCR, polymerase chain reaction; ss-DNA, single-stranded DNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate.

During our search for RT inhibitors active against the IIIB strain of HIV-1 mutants resistant to various NNRTIs, we observed that the AAP-BHAP U-104489 selects for the G190E mutation in cell culture (Olmsted *et al.*, 1996). Interestingly, the replication of the virus bearing the G190E mutation in its RT was retarded (Olmsted *et al.*, 1996). In an attempt to understand the biochemical basis for this phenomenon, we have characterized the enzymatic properties of the purified p66/p51 heterodimers of the G190A and G190E mutant enzymes. In this paper we demonstrate that the presence of glutamate at residue 190 in HIV-1 RT (G190E RT) impairs both the processive DNA synthesis and RNA degradation. The relevance of these results in regard to the reduced replication capacity of G190E-containing HIV-1 is discussed.

MATERIALS AND METHODS

Chemicals. General laboratory chemicals were purchased from Sigma Chemical Co. and Bio-Rad. Low-melting-point agarose, Taq polymerase, T4 ligase, restriction enzymes EcoRI and HindIII, and molecular weight markers were from Gibco/BRL. Proteinase K was from Boehringer Mannheim and DNase I was obtained from Ambion. The substrates poly(rA)-oligo(dT), poly(rA)-poly(dT), thymidine triphosphate (dTTP), chelating Sepharose, and RNA polymerase were purchased from Pharmacia/LKB Biotechnology Inc. The [³H]dTTP was obtained from DuPont-New England Nuclear and diluted with cold dTTP. The select-D spin column was from 5'-3', Inc. For DNA sequencing and end-labeling [³⁵S]dATP and [γ -³²P]ATP were purchased from New England Nuclear. The Sequenase version 2.0 sequencing kit was obtained from U.S. Biochemicals Corp. Oligonucleotide primers for PCR and primer extension were purchased from Genosys Biotechnologies, Inc. Recombinant HIV-1 protease was provided by Dr. A. G. Tomasselli, and the HIV-1 RT inhibitors U-104489 and U-90152S were supplied by Dr. D. L. Romero of Upjohn Laboratories.

Cloning and Expression of Mutants of HIV-1 RT. The hexa-His-tagged HIV-1 RT expression plasmid DE-5,2 (Sharma *et al.*, 1991) was used to generate site-specific point mutations G190A and G190E as described (Fan *et al.*, 1995; Olmsted *et al.*, 1996). Competent *Escherichia coli* strain JM 109 (G190A) or DH5 α (G190E) was transformed with the G190A and G190E RT constructs and selected for ampicillin resistance. Resulting colonies were screened for expression of the p66 HIV-1 RT mutant proteins upon induction with 1 mM IPTG at 0.4 OD₆₀₀ (Chattopadhyay *et al.*, 1992). The *E. coli* cells were pelleted and lysed by SDS, and the cell lysate was analyzed by SDS-12% PAGE (Laemmli, 1970). The selected clone was then sequenced by the dideoxy method using the Sequenase version 2.0 sequencing kit.

Purification and Characterization of Mutants of HIV-1 RT. The crude *E. coli* extract was prepared by processing 20–40 g of cell paste. The IMAC purification of the p66/p66 homodimers of G190A and G190E RT mutants was carried out essentially as reported (Fan *et al.*, 1995). For further purification, the p66/p66 homodimers were dialyzed overnight at 4 °C against buffer A (50 mM Tris, pH 7.2, containing 1 mM DTT, 1 mM EDTA, and 6% glycerol). The dialyzed protein solution was applied to a 2-mL bed

volume of ss-DNA-cellulose column equilibrated with buffer A at a flow rate of 0.3 mL/min. The column was washed with 5 bed volumes of buffer A until the A_{280} of the effluent returned to baseline, followed by 5 bed volumes of buffer A containing 80 mM NaCl. The desired protein was eluted in buffer A containing 200 mM NaCl (Muller *et al.*, 1991) and analyzed for purity by SDS-PAGE. The p66/p51 heterodimers of the G190A and G190E RT mutants were obtained by *in vitro* processing of the corresponding purified p66/p66 homodimers with HIV-1 protease, as described for the wild-type p66 HIV-1 RT (Chattopadhyay *et al.*, 1992). The resulting G190A RT and G190E RT heterodimers were purified in a single IMAC step, as described elsewhere (Chattopadhyay *et al.*, 1992).

Activity and Protein Assays. RNA-dependent DNA polymerase (RT) activity was determined with poly(rA)-oligo(dT) as the template-primer (Chattopadhyay *et al.*, 1992). One unit of RT activity is defined as the amount of enzyme required to incorporate 1 nmol of [³H]dTTP into the template-primer in 1 h at 37 °C. The specific RT activity of the wild-type heterodimeric HIV-1 RT was 48900 ± 1200 units/mg under defined assay conditions. For inhibition studies, assays were carried out in the presence and absence of inhibitors (Fan *et al.*, 1995). All assays were performed in triplicate and K_i values are reported as mean values from three independent assay. The RNase H activity was measured using [³H]poly(rA)-poly(dT) substrate (Evans *et al.*, 1994) and a HIV-1 gag-based [³H]RNA/DNA hybrid (Evans *et al.*, 1994) with slight modifications in the assay protocol. All the RNase H activity determinations were made using a final concentration of 8 mM MgCl₂ (pH 8.5) or 8 mM MnCl₂ (pH 8.0). After the reaction, the unreacted substrate was precipitated with 10% TCA. The released monomers or oligomers as [³H]nucleotides is a measure of the RNase H activity under defined conditions (Evans *et al.*, 1994). The final TCA concentration in the scintillation vial was 0.2%. All the reported values were corrected for background in the absence of any enzyme. One unit of RNase H activity is defined as 1 pmol of TCA-soluble radiolabeled adenylate released from the RNA/DNA hybrid in 1 h at 37 °C. The specific RNase H activity of the wild-type heterodimeric HIV-1 RT-associated RNase H was 813 ± 31 units/mg. The Bradford protein assay (Bradford, 1976) was used to determine protein concentration with BSA as a standard.

RT Primer-Extension Assay. The plasmid pSMP-15 (Evans *et al.*, 1994) was used for preparation of positive-strand HIV-1 gag RNA. A 400-nucleotide HIV-1 gag RNA transcript (HIV-1 gag positive strand 683–1084) was prepared by T7 RNA polymerase runoff transcription of HindIII-digested pSMP15 by using a commercially available transcription kit (Stratagene). Reactions (100 μL) containing 3 μg of HindIII-digested pSMP15 plasmid DNA, 10 units of T7 RNA polymerase, 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 10 mM DTT, and 250 μM each ATP, CTP, GTP, and UTP were incubated at 37 °C for 1 h. The DNA template was digested at the end of the reaction with 10 units of RNase-free DNase at 37 °C for 20 min and then treated with 10 μL of 2 mg/mL proteinase K at 37 °C for 30 min. The RNA sample was extracted with phenol/chloroform and precipitated by ethanol, and free nucleotides were removed by passing the sample through a Select-D spin column. The RNA was stored at -80 °C at a concentration of 2.2 μM in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. The HIV-1

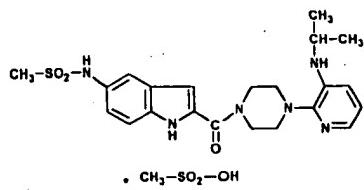
gag-specific primer Exo-2 (HIV-1 *gag* negative strand 785–765) was 5'-end-labeled with [³²P]ATP using T4 polynucleotide kinase. The *in vitro*-transcribed HIV-1 *gag* RNA template sequence and the DNA primer are as follows:

5' UCGACGCAGGACUCGGCUUGCUGAAGCGCCCGCACGGCAAGAGGCCAGGGCGAGGGCGACUGGUG
AGUACGCCAAAAUUUJGACUAGCGGAGCCUAAGGAGA-3'
3' GATCGCTCCGATCTTCCTCT-5' (Exo-2, 785–765)

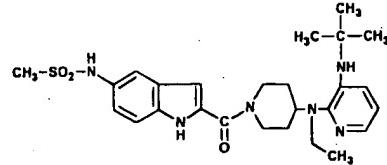
The preparation of template–primer (0.2 μM) and the RT primer-extension assay were according to DeStefano *et al.* (1994). Briefly, G190A RT or G190E RT (25 nM) was preincubated with template–primer for 1 min at 37 °C in 10 μL of 50 mM Tris-HCl, pH 8.0, 80 mM KCl, 1 mM DTT, and 0.1 mM EDTA. Extension of DNA was initiated by addition of MgCl₂, dNTPs, and with or without the enzyme trap in 2.5 μL of the above buffer to give a final concentration of 6 mM MgCl₂ and 50 μM each dATP, dCTP, dGTP, and dTTP. The enzyme trap used in the challenged RT primer-extension assays was 200 μg/mL unlabeled poly(rA)·oligo(dT). Reactions were stopped at indicated times with an equal volume of 2× gel loading buffer (80% formamide, 10 mM EDTA, pH 8.0, and 0.1% each xylene cyanol and bromphenol blue). Reaction mixtures were heat-denatured and analyzed by electrophoresis on 8% polyacrylamide–7 M urea gels (DeStefano *et al.*, 1994).

RNase H Cleavage Products by Denaturing Gel Electrophoresis. The RNase H substrate [³⁵S]poly(rA)·poly(dT) was prepared as described by Volkmann *et al.* (1993) using *E. coli* RNA polymerase. In a total volume of 300 μL, 20 μg of poly(dT) was incubated for 1 h at 37 °C with 50 units of *E. coli* RNA polymerase, 50 nM ATP, and 100 μCi of [α-³⁵S]-ATP in 50 mM Tris-HCl, pH 8.0, containing 100 mM KCl, 5 mM MgCl₂, 5 mM DTT, and 1 mM MnCl₂. After phenol extraction and ethanol precipitation, the hybrid was dried, dissolved in 100 μL of 10 mM Tris/HCl and 1 mM EDTA buffer, pH 8.0, and purified from unincorporated nucleotides by Select-D spin column. The RNA/DNA hybrid was used for determination of the degradation products of the RNase H reaction. Heterodimeric G190A RT or G190E RT (50 nM) in 50 μL of RNase H buffer (50 mM Tris-HCl, pH 8.5, 8 mM MgCl₂, 3% glycerol, 0.1 mg/mL BSA, and 0.02% NP-40) was incubated with 2 × 10⁶ cpm (0.3 μg) of [³⁵S]-poly(rA)·poly(dT) substrate at 37 °C. Aliquots (10 μL) of the reaction mixture were removed at 5-, 15-, 30-, and 60-min time points, mixed with 5 μL of formamide-containing stop solution. The samples were then heat-denatured and analyzed by electrophoresis on 10% denaturing polyacrylamide gels (Volkmann *et al.*, 1993).

Effect of a Challenger Substrate on RNA Degradation. The processive RNase H activity of G190E RT was examined by addition of excess unlabeled substrate after the onset of the reaction (Evans *et al.*, 1994). Briefly, heterodimeric wild-type HIV-1 RT (50 nM), G190A RT (50 nM), or G190E RT (250 nM) was incubated at 37 °C with 0.2 μg of [³H]poly(rA)·poly(dT) in a total volume of 90 μL of RNase H assay buffer (50 mM Tris-HCl, pH 8.5, 8 mM MgCl₂, 3% glycerol, 0.1 mg/mL BSA, and 0.02% NP-40). One minute after the onset of the reaction, 10 μL of unlabeled poly(rA)·poly(dT) (0.4 mg/mL) was added to give at least a 10-fold excess over labeled substrate. At 3-, 6-, and 15-min time points, 30 μL of the reaction mixture was removed and placed into a tube containing 160 μL of cold 10%



U-90152S



U-104489

FIGURE 1: Chemical structures of BHAP U-90152S and AAP-BHAP U-104489. The structure of U-90152S was published (Fan *et al.*, 1995).

trichloroacetic acid and 10 μL of 0.5 mg/mL salmon sperm DNA. All samples were processed and counted for RNase H activity as described (Evans *et al.*, 1994) for RNase H assay using [³H]poly(rA)·poly(dT).

The effect of the challenger substrate on RNA hydrolysis was also studied by following sizes of the RNase H cleavage products on denaturing polyacrylamide gels (Volkmann *et al.*, 1993). In this case, heterodimeric wild-type HIV-1 RT (50 nM), G190A RT (50 nM), or G190E RT (250 nM) was incubated at 37 °C with 0.3 μg of [³⁵S]poly(rA)·poly(dT). A 10-fold excess of unlabeled poly(rA)·poly(dT) was added 1 min after the onset of the reaction. The cleavage products were analyzed after 15 min of incubation at 37 °C with and without the challenger.

RESULTS AND DISCUSSION

Analysis of *E. coli* Expressed G190E HIV-1 RT. Recently we (Olmsted *et al.*, 1996) and others (Kleim *et al.*, 1993 and 1994) have shown that in cell culture the AAP-BHAP U-104489 (Figure 1) or the quinoxaline derivative S-2720 selects for the G190E resistance mutation. We also observed that the replication capacity of the virus bearing the G190E mutation in its RT was retarded (Olmsted *et al.*, 1996). In the present paper we have studied the effect of the G190E mutation on the enzymatic properties of p66/p66 HIV-1 RT. The G190E mutation in the clone selected for expression of G190E RT in *E. coli* was confirmed by sequencing of the entire RT and RNase H coding regions. The DNA sequencing data showed that the G190E is the only amino acid substitution in G190E RT.

As reported earlier for the wild-type HIV-1 RT (Chattopadhyay *et al.*, 1992), we performed single-step IMAC purification of p66/p66 homodimers from crude *E. coli* extracts containing G190E or G190A RT mutants. We observed that the specific RT activity of the IMAC-purified p66/p66 homodimer of G190E RT (7904 ± 83 units/mg) was about 23% compared to G190A RT (34155 ± 1103 units/mg). Subsequent affinity chromatography of IMAC-purified p66/p66 homodimer of G190E RT on a ss-DNA-cellulose column resulted in about a 4-fold increase in its specific activity (31215 ± 1052 units/mg). In contrast, the specific activity of the G190A RT was only increased 1.3-fold by further purification on the ss-DNA-cellulose. As

Table 1: Activity and Inhibition Characteristics of G190A and G190E RT Mutants^a

HIV-1 RT p66/p51	RT activity ^b (units/mg)	inhibition by		
		ddTTP ^c (K_i , nM)	U-104489 ^d (K_i , μ M)	U-90152S ^d (K_i , μ M)
wild type (G190)	48900 \pm 1200	298 \pm 15	0.13 \pm 0.01	0.37 \pm 0.003
G190A	49519 \pm 1309	313 \pm 12	0.12 \pm 0.006	0.06 \pm 0.001
G190E	30306 \pm 298	402 \pm 12	>100	3.32 \pm 0.65

^a Data represent mean \pm SD ($n = 3$). ^b One unit is defined as 1 nmol of labeled dTMP incorporated into poly(rA)-oligo(dT) in 1 h at 37 °C. ^c Calculated for competitive inhibition using a nonlinear least-squares method. ^d Calculated for noncompetitive inhibition using a nonlinear least squares method.

we have shown for the wild-type HIV-1 RT (Chattopadhyay *et al.*, 1992), purified homodimer of G190A RT and G190E RT on SDS-PAGE showed a 66-kDa polypeptide along the some minor bands of slightly lower molecular mass (data not shown). As described before (Chattopadhyay *et al.*, 1992) for wild-type HIV-1 RT, these relatively small polypeptides unrelated to p51 kDa polypeptide, we think, arise due to minor proteolysis of p66/p66 homodimer by bacterial enzymes. Therefore, we cannot rule out the possibility that the observed increase in the specific activity of G190E RT homodimer after ss-DNA-cellulose purification is the result of differences in associated microheterogeneity between the two enzymes.

Enzymatic Properties of p66/p51 Heterodimeric G190E RT. Since heterodimeric (p66/p51) HIV-1 RT is the relevant form of the enzyme *in vivo*, the p66/p66 homodimers were converted into p66/p51 heterodimers after *in vitro* processing with HIV-1 protease. The heterodimers were subsequently purified and were used in all the reported biochemical studies. The p66/p51 heterodimers of G190A RT and G190E RT mutants showed a 1:1 distribution of two bands corresponding to relative molecular masses of 66 and 51 kDa (data not shown). As noted previously for the wild-type heterodimer (Chattopadhyay *et al.*, 1992), the associated microheterogeneity observed with the corresponding p66/p66 homodimers disappeared upon HIV-1 protease treatment. Notably, purity of the p66/p51 heterodimers of both the mutant enzymes was comparable to the purity of our wild-type HIV-1 RT (Chattopadhyay *et al.*, 1992).

Table 1 shows a comparison of enzymatic properties of G190E RT with the wild-type (G190) or the G190A RT. The RT specific activity (Table 1) of the purified heterodimeric G190E RT is about 60% that of the wild-type RT or G190A RT. The DNA chain terminator, ddTTP, inhibited the RT activity of G190A and G190E enzymes in a competitive manner with K_i values of 313 \pm 12 and 402 \pm 12 nM, respectively (Table 1). Further inhibition studies with the purified G190E RT showed that it was highly resistant to the AAP-BHAP U-104489 (Table 1). However, the recombinant G190E RT, like U-104489-resistant HIV-1 (Olmsted *et al.*, 1996), was relatively less resistant to the BHAP (Figure 1) delavirdine (U-90152S). These *in vitro* studies with the G190E RT (Table 1) are consistent with the cell culture studies on U-104489-resistant HIV-1 containing the G190E substitution (Olmsted *et al.*, 1996).

Effect of the G190E Mutation on HIV-1 gag RNA-Directed DNA Synthesis. In an attempt to understand the basis for the relatively low specific RT activity of the G190E RT (Table 1), we carried out a primer-extension assay to examine

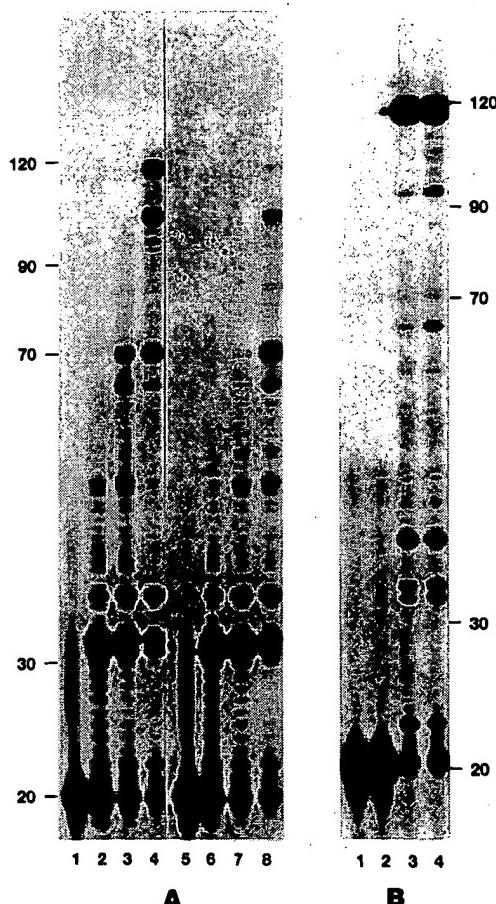


FIGURE 2: Effect of the G190E mutation on the length of primer extension during RNA-dependent DNA polymerization. The heteropolymeric HIV-1 gag-based template-primer used in the experiment is described under Materials and Methods. The primer was [³²P]-labeled 21-mer DNA. Samples were separated on a polyacrylamide-urea gel and visualized by autoradiography. The approximate sizes of extended products in nucleotides are indicated. (A) Lanes 1–4 represent primer-extension products during 0, 15, 30, and 60 s of incubation of [³²P]template-primer with G190A RT, respectively. Lanes 5–8 show products of primer-extension during 0, 15, 30, and 60 s of incubation of [³²P]template-primer with G190E RT, respectively. (B) Primer extension products made during the 10-min incubation at 37 °C with G190A RT (lane 3) and G190E RT (lane 4). Lanes 1 and 2 represent zero time controls for G190A RT and G190E RT, respectively.

DNA synthesis by G190E RT on an RNA template derived from the HIV-1 genome. A portion (683–785) of the *in vitro* transcribed HIV-1 gag RNA template sequence (683–1084) and the DNA primer used to assess processivity of RT polymerization are shown in the Materials and Methods section. This RNA was primed at position 785 with a [³²P]-5'-end-labeled 21-nucleotide-long DNA segment. Thus, full-length extension of the primer would result in a 120-nucleotide product containing 102 nucleotides (683–785) from the HIV-1 gag sequence and a short stretch of 18 nucleotides between the multiple cloning site and the T7 promoter. As shown in Figure 2A, there is a time-dependent elongation of the primer by the G190A RT (lanes 2–4). The final product of 120 nucleotides was synthesized in 60 s (Figure 2A, lane 4) by G190A RT or by wild-type HIV-1 RT (data not shown). On the other hand, at equivalent time points the products formed by the G190E RT were shorter (Figure 2A, lanes 6–8). As a result, the largest extension product synthesized by G190E RT was approximately 110

nucleotides (lane 8). The G190E RT synthesized more products in the 40–70-nucleotide range (lane 8) compared to the G190A RT (lane 4). In contrast to G190A RT (lane 4) or by wild-type HIV-1 RT (data not shown), the full-length extension product (120 nucleotides) was almost negligible within the first 60 s of incubation with G190E RT (lane 8), indicating an overall slower rate of polymerization for the G190E RT.

Although, compared to G190A RT, relatively less of the available primers were extended by G190E RT at the 1-min time point (Figure 2A), reactions halted at 10 min (Figure 2B, lanes 3 and 4) resulted in patterns of extension products which were similar for both the mutant enzymes. The relative intensity of the extended products (Figure 2B, lanes 3 and 4), including the major full-length product (120 nucleotides), was also comparable for both the enzymes. Moreover, patterns of primer extension products did not change with varying concentrations of G190A RT or G190E RT (data not shown). Thus, the full-length product in each case (Figure 2B, lanes 3 and 4) seems to result from multiple enzyme–substrate turnovers initiated at the potentially available primers.

Effect of the G190E Mutation on Processive DNA Synthesis. In order to determine the effect of the G190E mutation on processive DNA synthesis, the effect of a challenger substrate on DNA polymerization by G190E RT, G190A RT, and wild-type HIV-1 RT was examined (Figure 3). In this case, the enzyme is preincubated with the [³²P]template–primer and synthesis is then initiated by adding dNTPs and Mg²⁺ in the presence of 10-fold excess of the unlabeled poly(rA)·oligo(dT) used as the enzyme trap. The trap sequesters the enzyme molecules which have dissociated from the template–primer and prevents the trapped enzyme from rebinding to the [³²P]-labeled gag-based heteropolymeric template–primer. The extent of primer extension is then indicative of the relative effect of the G190E mutation on processive DNA synthesis.

As shown in Figure 3, presence of the challenger substrate decreases the ability of the G190E RT (Figure 3A,B, lanes 6) to synthesize the full-length product compared to the G190A RT (Figure 3A, lane 5) or wild-type HIV-RT (Figure 3B, lane 5). There is an abundance of primer-extension products by G190E RT that are smaller than 30 nucleotides (Figure 3A,B, lanes 6) relative to G190A RT (Figure 3A, lane 5) or wild-type HIV-1 RT (Figure 3B, lane 5). This suggests that the first round of extension by G190E RT reached these smaller oligonucleotides and the subsequent elongation of these intermediate products was blocked by the challenger trap. The presence of the trap in the preincubation control effectively sequestered wild-type HIV-1 RT, G190A RT, or G190E RT as shown by a lack of any primer-extension products (data not shown). Taken together, these results (Figure 3) demonstrate that the G190E RT, compared to G190A RT or wild-type HIV-1 RT, is relatively less processive during RNA-dependent DNA polymerization.

Effect of G190E Mutation on RT-Associated RNase H Activity. The negative effect of the G190E mutation on its RNase H activity, compared to wild-type RT or G190A RT, was an unexpected finding (Table 2). The G190E RT exhibited only 20% and 47% of the Mg²⁺-dependent RNase H activity of the G190A RT with the poly(rA)·poly(dT) and HIV-1 gag-based RNA/DNA hybrid, respectively. The Mn²⁺-dependent RNase H activity of G190E RT, compared

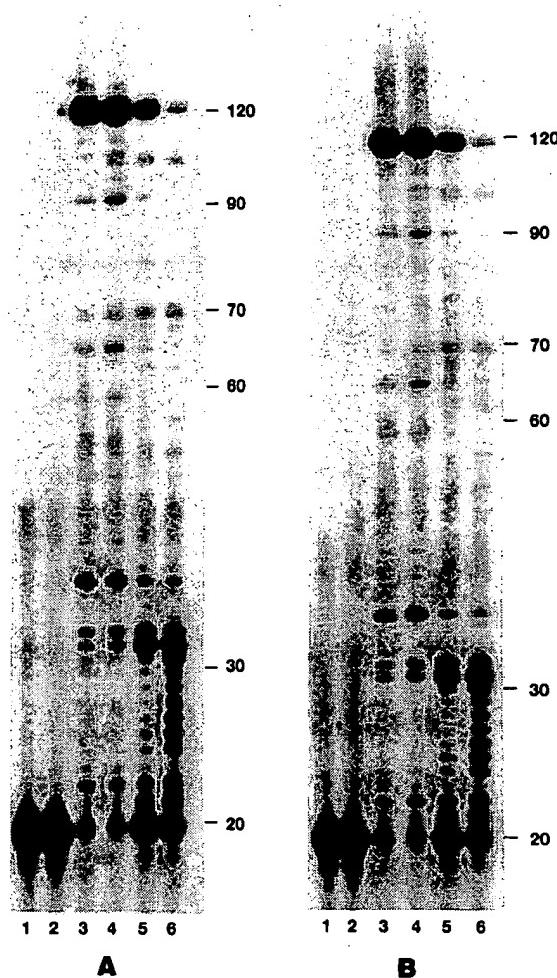


FIGURE 3: Effect of a trapping template–primer [poly(rA)·oligo(dt)] on the length of primer extension during RNA-dependent DNA polymerization by G190A RT, wild-type HIV-1 RT, and G190E RT. Samples were separated on a polyacrylamide–urea gel and visualized by autoradiography. The heteropolymeric HIV-1 gag-based template–primer was preincubated with wild-type HIV-1 RT, G190A RT or G190E RT and DNA synthesis was initiated by the addition of MgCl₂ and dNTPs in the presence and absence of the trapping polymer [poly(rA)·oligo(dt)]. Reactions were terminated with 2× loading buffer 10 min after initiation. (A) Zero time controls for G190A RT (lane 1) and G190E RT (lane 2); primer extension products made during the 10-min incubation at 37 °C with G190A RT (lane 3) and G190E RT (lane 4); effect of the excess challenger on primer-extension products during the 10-min incubation at 37 °C with G190A RT (lane 5) and G190E RT (lane 6). (B) Zero time controls for wild-type HIV-1 RT (lane 1) and G190E RT (lane 2); primer extension products made during the 10-min incubation at 37 °C with wild-type HIV-1 RT (lane 3) and G190E RT (lane 4); effect of the excess challenger on primer-extension products during the 10-min incubation at 37 °C with wild-type HIV-1 RT (lane 5) and G190E RT (lane 6). The approximate sizes of extended products in nucleotides are indicated.

to that of G190A RT, was about 60% with both of these substrates (data not shown). These results are consistent with studies (Evans *et al.*, 1994; Zhan *et al.*, 1994) that demonstrate that the RNase H activity of p66/p51 HIV-1 RT depends upon the nature of the RNA/DNA hybrid as well as the metal ion activator. The RNase H activity assays used in the above experiments measure the release of TCA-soluble [³H]oligonucleotides (<10 nucleotides) from [³H]poly(rA)·poly(dT) (Evans *et al.*, 1994). To further confirm the apparent low RNase H activity of G190E HIV-1 RT with

Table 2: RT-Associated RNase H Activities of G190A and G190E RT Mutants

HIV-1 RT p66/p51	[³ H]poly(rA)·poly(dT) ^a (units/mg)	HIV-1 gag-based [³ H]RNA/DNA ^a (units/μg)
G190A	932 ± 31 (100%)	11775 ± 376 (100%)
G190E	178 ± 28 (20%)	5541 ± 120 (47%)

^a Data represent mean ± SD ($n = 3$) for Mg^{2+} -dependent RNase H activity. One unit is defined as 1 pmol of TCA-soluble [³H] monomers or oligomers released from the RNA/DNA hybrid in 1 h at 37 °C. The percent activity for G190E RT relative to G190A RT (100%) is given in parentheses. Values reported for the G190A RT were comparable to those for the wild-type HIV-1 RT (not shown).

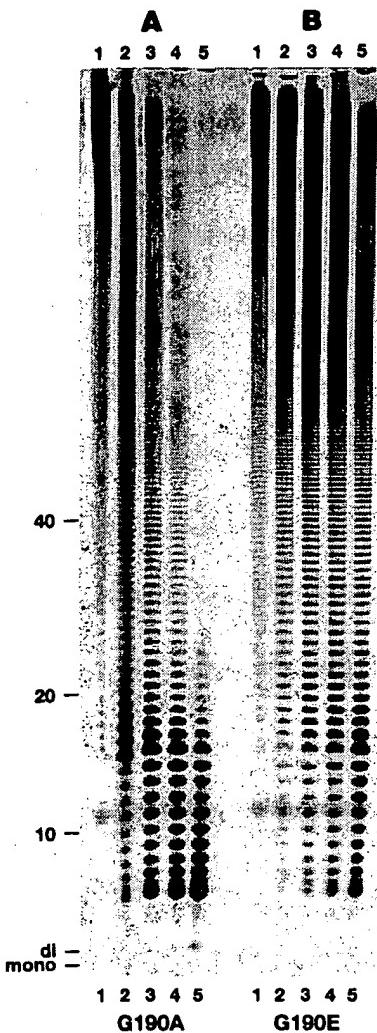


FIGURE 4: Determination of the sizes of the products of Mg^{2+} -dependent RNase H digestion of [³⁵S]poly(rA)·poly(dT) by G190A RT (panel A) and G190E RT (panel B). Lanes 1–5 represent 0-, 5-, 15-, 30-, and 60-min time points of incubation at 37 °C. The approximate sizes of RNA cleavage products in nucleotides are indicated.

[³H]poly(rA)·poly(dT) (Table 2), the size distribution of products generated by RNase H was determined.

As shown in Figure 4, the time-dependent size distribution of products generated by G190E RT-associated RNase H from [³⁵S]poly(rA)·poly(dT) substrate in the presence of Mg^{2+} differed markedly from that of the G190A RT-associated RNase H. The cleavage by G190A RT resulted in an initial formation of oligonucleotides smaller than 15

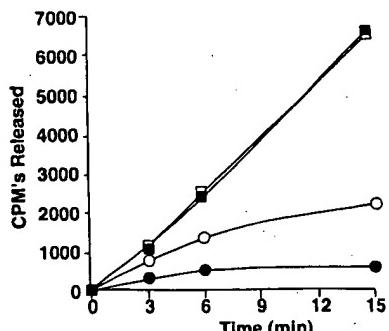


FIGURE 5: Effect of challenger substrate on Mg^{2+} -dependent degradative activity of G190A (50 nM) and G190E (250 nM) HIV-1 RT-associated RNase H. After 1 min at 37 °C, excess challenger or water was added to samples. At the times indicated, aliquots were withdrawn and the reaction was quenched for assaying RNase H activity expressed as cpm released from [³H]poly(rA)·poly(dT). (□) G190A RT; (■) G190E RT; (○) G190A RT in the presence of challenger substrate; (●) G190E RT in the presence of challenger substrate.

nucleotides in length within 5 min of incubation (panel A, lane 2). This was followed by the rapid appearance of smaller oligonucleotides 5–10 nucleotides in length at the 15-min point (panel A, lane 3). The formation of these small size oligonucleotides continued to increase with time (panel A, lanes 4 and 5), suggesting that smaller oligonucleotides arise from the larger ones by endonuclease RNase H activity. Our results are consistent with an earlier report (Zhan *et al.*, 1994) suggesting that Mg^{2+} -dependent RT-associated RNase H activity is primarily responsible for the hydrolysis of the [³⁵S]poly(rA)·poly(dT).

In contrast to G190A RT (Figure 4A) or wild-type HIV-1 RT (Fan *et al.*, 1996), cleavage by G190E RT did not result in accumulation of the smaller oligonucleotides (5–10 bases in length) to the same extent (Figure 4B, lanes 2–5). This was consistent with the presence of a relative abundance of large (> 15 bases) oligonucleotides (panel B, lanes 2–5), indicating impaired endonuclease RNase H activity of the G190E RT relative to that of G190A RT or wild-type HIV-1 RT (Fan *et al.*, 1996). Thus, the observed differences in the Mg^{2+} -dependent RNase H activities between the G190A and G190E enzymes (Table 2) correlates very well with the observed differences in sizes of the small products released from [³⁵S]poly(rA)·poly(dT) substrate (Figure 4). The pattern of Mn²⁺-dependent RNase H cleavage products (data not shown) generated from [³⁵S]poly(rA)·poly(dT) was also consistent with low Mn²⁺-dependent RNase H activity of G190E RT compared to that of G190A RT.

Effect of the G190E Mutation on Processive RNA Degradation. Previously, we have compared relative processivities of wild-type HIV-1 RT with a p15 RNase H domain by studying RNA degradation activity of the [³H]poly(rA)·poly(dT) duplex in the presence of a challenger substrate (Evans *et al.*, 1994). Therefore, we investigated the effect of the challenger substrate poly(rA)·poly(dT) on the release of oligomers from [³H]poly(rA)·poly(dT) by Mg^{2+} -dependent RT-associated RNase H activities of the G190A and G190E mutant enzymes (Figure 5). Since the G190E RT, compared to G190A RT or wild-type HIV-1 RT, has 5-fold reduced RNase H activity with this substrate (Table 2), a 5-fold excess of G190E RT was used. In contrast to G190A RT, the G190E RT cleaved the RNA/DNA hybrid in a relatively less processive manner in the presence of the challenger substrate

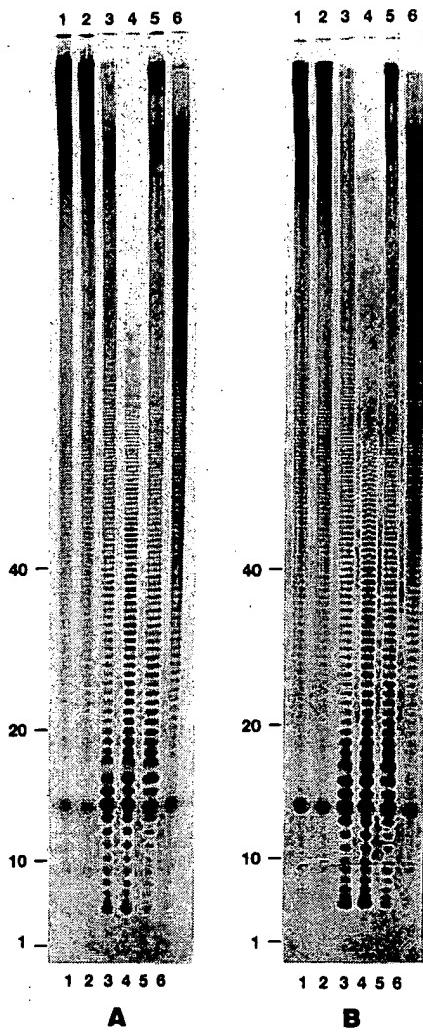


FIGURE 6: Effect of challenger substrate on the sizes of the products of Mg^{2+} -dependent RNase H digestion of [^{35}S]poly(rA)-poly(dT) by G190A RT (50 nM), wild-type HIV-1 RT (50 nM), or G190E RT (250 nM). (A) Zero time points for G190A RT (lane 1) and G190E RT (lane 2); degradation products after 15 min of digestion by G190A RT (lane 3) and G190E RT (lane 4); effect of challenger substrate on degradation products after 15 min of digestion by G190A RT (lane 5) and G190E RT (lane 6). (B) Zero time points for wild-type HIV-1 RT (lane 1) and G190E RT (lane 2); degradation products after 15 min of digestion by wild-type HIV-1 RT (lane 3) and G190E RT (lane 4); effect of challenger substrate on degradation products after 15 min of digestion by wild-type HIV-1 RT (lane 5) and G190E RT (lane 6). The appropriate sizes of RNA cleavage products in nucleotides are indicated.

(Figure 5). As shown, differences in RNase H activities between the two enzymes, in the presence of a challenger, were observed at each time point.

The above results were further substantiated from the analysis of sizes of the cleavage products by wild-type HIV-1 RT and both the mutant enzymes in the presence of an unlabeled challenger substrate (Figure 6A,B, lanes 5 and 6). In the controls without the challenger substrate (Figure 6A,B, lanes 3 and 4), all the enzymes displayed similar [^{35}S]-labeled oligomeric (<15 nucleotides) cleavage products when the G190E RT concentration was 5-fold higher than the G190A RT or wild type HIV-1 RT. However, as shown in Figure 6A,B (lane 6), the presence of the challenger substrate decreased the RNase H-mediated hydrolysis of the [^{35}S]poly(rA)-poly(dT) by G190E RT. There was very little release

of small (<15) nucleotides by G190E RT, compared to that of G190A RT (Figure 6A, lane 5) or wild-type HIV-1 RT (Figure 6B, lane 5), indicating that G190E RT was effectively sequestered by the challenger trap after its dissociation from the substrate. It is concluded (Figure 6) that G190E RT, compared to G190A RT (Figure 6A, lane 5) or wild-type HIV-1 RT (Figure 6B, lane 5), is less efficient in processive RNA degradation. Together, the impairment in DNA synthesis (Table 1, Figures 2 and 3) and RNA degradation (Table 2, Figures 4 and 6) by G190E RT shows the deleterious effect of a drug-resistance mutation (G190E) on two distinct functional activities of HIV-1 RT. These results are consistent with our virology studies regarding diminished replication capacity of G190E-bearing HIV-1 resistant to the AAP-BHAP U-104489 (Olmsted *et al.*, 1996).

The G190E resistance mutation has also been observed in HIV-1 infected cells treated with a quinoxaline derivative, S-2720 (Kleim *et al.*, 1993). Accordingly, Kleim *et al.* (1994) have expressed the G190E RT mutant in *E. coli* as a fusion protein containing the maltose-binding domain as the affinity tag. A dramatic decrease (96%) in RT activity was observed, suggesting that glutamate substitution at residue 190 in HIV-1 RT is responsible for this loss of activity. It was not known if the isolated protein was pure with regard to other protein contaminants including the inactive RT protein. Therefore, the possibility exists that the extremely low RT activity (4%) for the G190E enzyme may be due to the partially purified nature of the G190E RT (Kleim *et al.*, 1994). Furthermore, Larder *et al.* (1987) reported *E. coli* expression of a HIV-1 RT mutant bearing Arg at 190 (G190R) and showed that its polymerase activity in crude *E. coli* extracts was about 23% of the RT activity observed for the wild-type enzyme. Unlike our present studies, these mutant enzymes were not produced by specific processing of the p66/p66 homodimers with HIV-1 protease. Thus, in the absence of any biochemical analysis of purified G190E RT or G190R RT, the previously reported (Larder *et al.*, 1987; Kleim *et al.*, 1994) 4–23% RT activity of these mutant enzymes remained unexplained.

Both homodimeric and heterodimeric HIV-1 RT are known to bind single-stranded DNA (Bakhanashvili & Hizi, 1994) and can be purified to homogeneity by affinity chromatography on ss-DNA-cellulose columns (Muller *et al.*, 1991). In our hands, a second affinity purification on ss-DNA-cellulose columns of homodimers of both the mutant enzymes showed comparable purity when analyzed by SDS-PAGE (data not shown). Furthermore, the isolated p66/p66 homodimer of G190E RT, like the p66/p66 homodimer of wild-type HIV-1 RT (Chattopadhyay *et al.*, 1992) or G190A RT, was fully competent to undergo complete transformation to a stable heterodimer (p66/p51) in the presence of HIV-1 protease. However, the purified p66/p51 heterodimer of G190E RT showed relative specific RT activity which was 60% relative to that of the G190A RT or wild-type HIV-1 RT (Table 1). One possible explanation is that this difference arises due to the presence of 60% active protein in p66/p51 G190E RT compared to the p66/p51 G190A RT. However, this interpretation is not supported by results from the effect of the challenger substrate in the RT primer-extension assay. As shown in Figure 3, there was a significant impairment in the G190E RT function with regard to processive DNA synthesis (lanes 5 and 6) under conditions where wild-type HIV-1 RT,

G190A RT, and G190E RT exhibited similar RT activities (lanes 3 and 4 of Figure 3A,B). Likewise, the observed decrease in the RNase H activity of the G190E RT (Table 2) is unlikely to be due to inactivation of its RT-associated RNase H during purification. The challenger assays (Figures 5 and 6), performed under adjusted conditions in which all the enzymes display similar RNase H activities, showed a time-dependent decrease in RNase H activity and an insignificant release of small (<15 nucleotides) cleavage products by G190E RT relative to G190A RT or wild-type HIV-1 RT.

Other groups have shown that during *in vitro* processive DNA synthesis, HIV-1 RT faces template nucleotide positions at which continued extension is difficult (Abbotts *et al.*, 1993; Klarmann *et al.*, 1993). The probability of RT dissociation from these pause sites is relatively high (Abbotts *et al.*, 1993; Klarmann *et al.*, 1993). Accordingly, one would expect a time-dependent accumulation of product molecules of corresponding length. Since mechanisms producing these blocks in processive DNA synthesis are not well understood, we cannot rule out the possibility that some of the shorter products we observed during our studies (Figure 3) are due to the presence of pause sites in the RNA template.

Overall, our results support the conclusion that G190E RT is less processive compared to G190A RT or wild-type HIV-1 RT with regard to both polymers and RNase H functions. In addition, they indicate that the G190E RT, compared to G190A RT or wild-type HIV-1 RT, dissociates more frequently from the substrate during DNA synthesis or RNA hydrolysis (Figures 3 and 6). There are other mechanisms that may also contribute to the decrease in processivity of the G190E RT. For example, factors that lower the rate of nucleotide incorporation also decrease the processivity of the RT on natural sequence or homopolymeric templates (DeStefano *et al.*, 1992). However, this is a remote possibility for the relatively low processivity of G190E RT since the U-104489-resistant HIV-1 (Olmsted *et al.*, 1996) is sensitive to AZT ($IC_{50} = 0.01 \mu M$). Moreover, the chain terminator ddTTP competitively inhibits the G190E RT similarly to G190A RT (Table 1). Another possible mechanism for decreased processivity of G190E RT relative to G190A RT or wild-type HIV-1 RT is that pause sites, if present in our HIV-1 gag RNA template, could be barriers to *in vitro* polymerization by G190E RT.

The mechanism by which the G190E mutation induces resistance toward NNRTIs is unclear. According to the three-dimensional structure of HIV-1 RT complexed with nevirapine (Kohlstaedt *et al.*, 1992) or other NNRTIs (Ren *et al.*, 1995), the binding pocket also contains G190, which is a part of the antiparallel β sheet containing Tyr-181 and Tyr-188. The substitution of Gly with Glu in G190E RT may affect the flexibility of the β -strand with the consequence of diminished affinity for NNRTIs that interact with the G190 amino acid. The observed higher IC_{50} values for most of the NNRTIs (Kleim *et al.*, 1994) support this hypothesis.

To our knowledge, this is the first example of a resistance mutation (G190E) that results in an impairment of two functions of HIV-1 RT. Since RNA-dependent DNA polymerization and RNase H-mediated degradation of the RNA template are fundamental to viral replication, our findings imply that a defect in these functions of G190E RT is most likely involved in the retarded *in vitro* replication capacity of the AAP-BHAP-(U-104489) resistant HIV-1 (Olmsted *et*

al., 1996). The future potential of such NNRTIs, in delaying drug resistance with long-term benefit to HIV-1 infected patients, remains to be addressed.

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REFERENCES

- Abbotts, J., Bebenek, K., Kunkel, T. A., & Wilson, S. H. (1993) *J. Biol. Chem.* 268, 10312–10323.
- Bacolla, A., Shih, C.-K., Rose, J. M., Piras, G., Warren, T. C., Grygon, C. A., Ingraham, R. H., Cousins, R. C., Greenwood, D. J., Richman, D., Cheng, Y.-C., & Griffin, J. A. (1993) *J. Biol. Chem.* 268, 16571–16577.
- Balzarini, J., Perez-Perez, M.-J., San Felix, A., Schols, D., Perno, C.-F., Vandamme, A.-M., Camarasa, M.-J., & De Clercq, E. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 4392–4396.
- Balzarini, J., Karlsson, A., Perez-Perez, M.-J., Vrang, L., Walbes, J., Zhang, H., Oberg, B., Vandamme, A.-M., Camarasa, M.-J., & De Clercq, E. (1993) *Virology* 192, 246–253.
- Bakharashvili, M., & Hizi, A. (1994) *Biochemistry* 33, 12222–12228.
- Boyer, P. L., Currens, M. J., McMahon, J. B., Boyd, M. R., & Hughes, S. H. (1993) *J. Virol.* 67, 2412–2420.
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254.
- Chattopadhyay, D., Evans, D. B., Deibel, M. R., Vosters, A. F., Eckenrode, F. M., Einsphar, H. M., Hui, J. O., Tommasselli, A. G., Zurcher-Neely, H. A., Heinrikson, R. L., & Sharma, S. K. (1992) *J. Biol. Chem.* 267, 14227–14232.
- Coffin, J. M. (1995) *Science* 267, 483–489.
- DeClercq, E. D. (1994) *Biochem. Pharmacol.* 47, 155–169.
- DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Fay, P. J., & Bambara, R. A. (1992) *Biochim. Biophys. Acta* 1131, 270–280.
- DeStefano, J. J., Mallaber, L. M., Fay, P. J., & Bambara, R. A. (1994) *Nucleic Acids Res.* 22, 3793–3800.
- De Vreese, K., Debyser, Z., Vandamme, A.-M., Pauwels, R., Desmyter, J., De Clercq, E., & Anne, J. (1992) *Virology* 188, 900–904.
- Dueweke, T. J., Kézdy, F. J., Waszak, G. A., Deibel, M. R., Jr., & Tarpley, W. G. (1992) *J. Biol. Chem.* 267, 27–30.
- Evans, D. B., Fan, N., Swaney, S. M., Tarpley, W. G., & Sharma, S. K. (1994) *J. Biol. Chem.* 269, 21741–21747.
- Fan, N., Evans, D. B., Rank, K. B., Thomas, R. C., Tarpley, W. G., & Sharma, S. K. (1995) *FEBS Lett.* 359, 233–238.
- Fan, N., Rank, K. B., Poppe, S. M., Tarpley, W. G., & Sharma, S. K. (1996) *Biochemistry* 35, 1911–1917.
- Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., & Markowitz, M. (1995) *Nature* 373, 123–126.
- Klarmann, G. J., Schauber, C. A., & Preston, B. D. (1993) *J. Biol. Chem.* 268, 9793–9802.
- Kleim, J.-P., Bender, R., Billhardt, U.-M., Meichsner, C., Riess, G., Rosner, M., Winkler, I., & Paessens, A. (1993) *Antimicrob. Agents Chemother.* 37, 1659–1664.
- Kleim, J.-P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., & Riess, G. (1994) *Virology* 200, 696–701.
- Kohlstaedt, L. A., Wang, J. A., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1992) *Science* 256, 1783–1790.
- Laemmli, U. K. (1970) *Nature* 227, 680–685.
- Larder, B. A., Purifoy, D. J. M., Powell, K. L., & Darby, G. (1987) *Nature* 327, 716–717.
- Loya, S., Bakhanashvili, M., Tal, R., Hughes, S. H., Boyer, P. L., & Hizi, A. (1994) *AIDS Res. Hum. Retroviruses* 10, 939–946.
- Mellors, J. W., Dutschman, G. E., Im, G.-J., Tramontano, E., Winkler, S. R., & Cheng, Y.-C. (1992) *Mol. Pharmacol.* 41, 446–451.
- Muller, B., Restle, T., Kuhnel, H., & Goody, R. S. (1991) *J. Biol. Chem.* 266, 14709–14713.
- Nanni, R. G., Ding, J., Jacobo-Molina, A., Hughes, S. H., & Arnold, E. (1993) *Perspect. Drug Discovery Des.* 1, 129–150.

Effect of a Mutation on RT and RNase H Functions

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- Nunberg, J. H., Schleif, W. A., Boots, E. J., O'Brien, J. A., Quintero, J. C., Hoffman, J. M., Emini, E. A., & Goldman, M. E. (1991) *J. Virol.* 65, 4887–4892.
- Olmsted, R. A., Slade, D. E., Kopta, L. A., Poppe, S. M., Poel, T. J., Yagi, Y., Romero, D. L., Rank, K. B., Sharma, S. K., Thomas, R. C., & Tarpley, W. G. (1996) *J. Virol.* 70, 3698–3705.
- Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D., & Stammers, D. (1995) *Struct. Biol.* 2, 293–302.
- Richman, D., Shih, C.-K., Lowy, I., Rose, J., Prodanovich, P., Goff, S., & Griffin, J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 11241–11245.
- Richman, D. D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S. A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., Shih, C.-K., Myers, M., & Griffin, J. (1994) *J. Virol.* 68, 1660–1666.
- Saag, M. S., Emilio, E. A., Laskin, O. L., Douglas, J., Lapidus, W., Schleif, W. A., Whitley, R. J., Hildebrand, B. S., Byrnes, V. W., Kappes, J. C., Anderson, K. W., Massari, F. E., & Shaw, G. W. (1993) *New Engl. J. Med.* 329, 1065–1072.
- Sardana, V. V., Emini, E. A., Gotlib, L., Graham, D. J., Lineberger, D. W., Long, W. J., Schlabach, A. J., Wolfgang, J. A., & Condra, J. H. (1992) *J. Biol. Chem.* 267, 17526–17530.
- Schinazi, R., Larder, B., & Mellors, J. (1994) *Internat. Antiviral News* 2, 72–75.
- Sharma, S. K., Evans, D. B., Vosters, A. F., McQuade, T. J., & Tarpley, W. G. (1991) *Biotechnol. Appl. Biochem.* 14, 69–81.
- Sharma, S. K., Evans, D. B., Vosters, A. F., Chattopadhyay, D., Hoogerheide, J. G., & Campbell, C. M. (1992) *Methods: Companion Methods Enzymol.* 4, 57–67.
- Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, K. A., Chirino, A. J., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 3911–3915.
- Taylor, P. B., Culp, J. S., Debouck, C., Johnson, R. K., Patil, A. D., Woolf, D. J., Brooks, I., & Hertzberg, R. P. (1994) *J. Biol. Chem.* 269, 6325–6331.
- Volkmann, S., Wohr, B. G., Tisdale, M., & Moelling, K. (1993) *J. Biol. Chem.* 268, 2674–2683.
- Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S., & Shaw, G. M. (1995) *Nature* 373, 117–122.
- Wu, J. C., Warren, T. C., Adams, J., Proudfoot, J., Skiles, J., Raghavan, P., Perry, C., Potocki, I., Farina, P. R., & Grob, P. M. (1991) *Biochemistry* 30, 2022–2026.
- Zhan, X., Tan, C.-K., Scott, W. A., Mian, A. M., Downey, K. M., & So, A. (1994) *Biochemistry* 33, 1366–1372.

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